



UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JOSY GOLDONI LAZARINI

**Prospecção da atividade anti-inflamatória de *Eugenia leitonii* D. Legrand, uma
fruta nativa Brasileira**

**Prospection of the anti-inflammatory activity of *Eugenia leitonii* D. Legrand, a
Brazilian native fruit**

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Dissertação apresentada à Faculdade de Odontologia de
Piracicaba da Universidade Estadual de Campinas, para
obtenção do título de Mestre em Odontologia, na Área de
Farmacologia, Anestesiologia e Terapêutica.

Orientador: Prof. Dr. Pedro Luiz Rosalen

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RESUMO

Na busca por substâncias alternativas com atividade biológica, as espécies frutíferas nativas do Brasil demonstram potencial funcional, devido principalmente à presença de compostos fenólicos como flavonoides e antocianinas que apresentam em sua composição. Estudos realizados pelo nosso grupo de pesquisa relataram partes (semente, folha e polpa) das espécies de *Eugenia* (*E. brasiliensis*, *E. uniflora*, *E. leitonii* e *E. myrcianthes*) exibiram atividades antioxidante e anti-inflamatória. Em adição, a semente da espécie *E. leitonii* exibiu o melhor efeito na inibição da migração de neutrófilos na cavidade peritoneal em camundongos. Desta forma, dando continuidade à investigação de frutas nativas brasileiras pouco exploradas, os objetivos deste estudo foram avaliar a atividade anti-inflamatória, mecanismo(s) de ação e o perfil fitoquímico do extrato de sementes de *Eugenia leitonii*. O extrato hidroetanólico (80:20, v/v) da semente de *E. leitonii* (EL) foi avaliado por meio dos testes de viabilidade celular, determinação de mediadores inflamatórios (TNF- α , IL-1 β and CXCL2/MIP-2) e ativação de NF- κ B em células RAW 264.7. O extrato de EL também foi avaliado *in vivo*, quanto aos níveis de mediadores inflamatórios (TNF- α , IL-1 β and CXCL2/MIP-2) no lavado peritoneal; ensaio de migração de neutrófilos induzido por carragenina; avaliação da expressão de moléculas de adesão (ICAM-1) e rolamento (P-selectina) no endotélio vascular e edema de pata induzido por carragenina. Por fim, foi quantificado o conteúdo total de compostos fenólicos e identificados por LC-MS/MS. Os resultados mostraram que o extrato de EL não alterou a viabilidade celular significativamente ($P > 0,05$) nas concentrações de 0,02 a 200 $\mu\text{g mL}^{-1}$; em 200 $\mu\text{g mL}^{-1}$ reduziram os níveis de TNF- α e CXCL2/MIP-2 e a ativação de NF- κ B em células RAW 264.7 ($P < 0,05$). Os camundongos tratados via oral na dose de 300 mg kg^{-1} com extrato de EL apresentaram reduções significativas dos níveis de TNF- α e CXCL2/MIP-2, também apresentaram diminuições nas expressões das moléculas de adesão (ICAM-1) e rolamento (P-selectinas) comparados ao controle negativo carragenina ($P < 0,05$). Os animais tratados nas doses de 30, 100 e 300 mg kg^{-1} tiveram reduções na migração de neutrófilos induzido por carragenina comparado ao controle carragenina ($P < 0,05$) e finalmente, os animais tratados oralmente com o extrato de EL na dose de 300 mg kg^{-1} apresentaram redução máxima no ensaio de edema de pata induzido por carragenina na 3ª hora (64 %), comparado ao controle carragenina ($P < 0,05$). O extrato de EL apresentou um total de $158,74 \pm 3,5$ mg AG/g e foram identificados compostos fenólicos como flavonoides e antocianinas, por meio da análise de LC-MS/MS. Assim, essa espécie genuinamente brasileira encontrada na mata atlântica, um ecossistema ameaçado, demonstrou ser uma promissora fonte de

compostos bioativos com potencial anti-inflamatório, atuando sobre vias de mediadores inflamatórios envolvendo moléculas de adesão e rolamento, e demonstrou efeito anti-edematogênico no edema de pata. O extrato de EL exibiu um teor de compostos fenólicos (flavonoides e antocianinas) maior do que aos frutos tradicionais, e que podem estar relacionados com o efeito anti-inflamatório observado possibilitando seu uso no enriquecimento de alimentos.

Palavras chave: *Eugenia leitonii*, anti-inflamatório, fruta nativa.

ABSTRACT

In the search for alternative bioactive molecules, Brazilian native fruit species have demonstrated functional potential due to the presence of phenolic compounds such as flavonoids, anthocyanins and others in their composition. Previous studies performed by our research group found that parts (leaves, seeds and pulps) of the *Eugenia* species (*E. brasiliensis*, *E. uniflora*, *E. leitonii* e *E. myrcianthes*) have antioxidant and anti-inflammatory activity. In addition the seeds of *E. leitonii* showed better activity in decrease of neutrophil influx into the peritoneal cavity of mice. Thus, continuing the investigation of unexplored Brazilian native fruits, this study evaluated the anti-inflammatory activity, mechanism(s) of action and phytochemical profile of the extract from the seeds of *E. leitonii*. The hydroethanolic extract (80:20, v/v) of *E. leitonii* seeds (EL) was evaluated by means of tests assessing cytotoxicity, modulation of inflammatory mediators (TNF- α , IL-1 β and CXCL2/MIP-2) and NF- κ B activation in RAW 264.7 macrophage cells. The EL extract was assessed *in vivo* concerning the inflammatory mediators levels (TNF- α , IL-1 β and CXCL2/MIP-2) in the peritoneal fluid of the animals; the influx of neutrophil migration into their peritoneal cavity; evaluation of ICAM-1 and P-selectin expression in vascular endothelium; and carrageenan-induced paw edema. Finally, the total phenolic content of the extract was measured and phenolic compounds were identified by LC-MS/MS. The results showed that EL extract did not affect significantly ($P > 0.05$) cell viability at the concentrations ranging from 0.02 to 200 mg μ l⁻¹; at 200 mg μ l⁻¹ reduced the levels of TNF- α and CXCL2/MIP-2, and NF- κ B activation in RAW 264.7 cells ($P < 0.05$). Mice pre-treated orally with EL extract (300 mg kg⁻¹) showed significantly decreased levels of TNF- α and CXCL2/MIP-2, also there was a decrease in ICAM-1 and P-selectin expression as compared with the control group ($P < 0.05$). The mice pretreated at doses 30, 100 e 300 mg kg⁻¹ showed decrease of the influx of neutrophils into the peritoneal cavity of the mice as compared with the negative control group carrageenan ($P < 0.05$) and finally, the mice pretreated orally with EL extract at 300 mg kg⁻¹ demonstrated maximum reduction of edema at 3 h (64 %) when compared with carrageenan ($P < 0.05$). The EL extract showed total phenolic contents yielded 158.74 $\pm$ 3.5 mg GAE/g and flavonoids and anthocyanins were identified by LC-MS/MS. Thus, this genuine Brazilian species founded in Atlantic Forest, an ecosystem threatened, showed to be a promising source of bioactive compounds with anti-inflammatory potential, acting on pathways of inflammatory mediators involving rolling and adhesion molecules and showed anti-edematogenic effect in the paw edema. The EL extract exhibited content of phenolic

compounds (flavonoids and anthocyanins) higher than that of traditional fruits, which may be related with the anti-inflammatory effects observed allowing its use to enrich foods .

Keywords: *Eugenia leitonii*, anti-inflammatory, native fruit.

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1 INTRODUÇÃO

Historicamente, os produtos naturais têm sido utilizados na medicina popular com base no conhecimento empírico adquirido ao longo de várias gerações. A eficácia de produtos naturais sobre diversas condições patológicas é amplamente descrita na literatura, sendo estes a principal fonte de novas drogas disponibilizadas no mercado como, por exemplo, anti-inflamatórios (ácido salicílico), anti-hipertensivos (captopril), quimioterápicos (vincristina, vimblastina e taxol), dentre outras (Viegas e Bolzani, 2006).

De acordo com Newman e Cragg (2012), no período de 1981 a 2010, 50% dos novos fármacos descobertos e aprovados pela FDA (Food and Drug Administration) foram produtos naturais ou derivados dos mesmos. Esses dados não apenas fortalecem a pesquisa científica neste setor, como também aumentam os desafios dos pesquisadores em identificar novas moléculas, elucidar o(s) seu(s) mecanismo(s) de ação e propor o uso terapêutico.

Estima-se que 22% da biodiversidade do mundo esteja no Brasil e, dentre os diversos locais, as florestas tropicais abrigam um grande número de espécies de flora que possuem substâncias com complexidade química e atividade biológica (Ferro et al., 2006). Na busca por novas moléculas bioativas, as espécies frutíferas nativas brasileiras inseridas nessa rica biodiversidade são um novo alvo a ser explorado, uma vez que apresentam potencial agroindustrial, alimentício e terapêutico. Estas espécies despertam o interesse da comunidade internacional, particularmente em países desenvolvidos, com recente destaque na pauta da programação da BBC de Londres¹, pois é relevante a busca de novas substâncias bioativas de produtos naturais e/ou a procura por alimentos funcionais que melhorem a qualidade de vida. Nos Estados Unidos, há centros de pesquisa que investigam o uso/consumo de alimentos (frutas), inclusive as brasileiras, em alvos fisiológicos e farmacológicos com finalidade de verificar a ação neutralizadora dos componentes da dieta sobre os radicais livres, que levam a consequentes doenças crônicas, cuja prevenção e tratamento tem sido um grande desafio para a medicina como a hipertensão, obesidade, osteoporose, artrite e outras. Estes alimentos, uma vez reconhecidos seu potencial bioativo no organismo, têm sido definidos como alimentos funcionais ou nutracêuticos (Vidal et al., 2012).

No Brasil, a maioria das frutas nativas ainda permanecem inexploradas e são comumente desvalorizadas e negligenciadas, tanto na comercialização quanto no seu consumo. Esta falta de incentivo e do interesse brasileiro não se restringe apenas à produção

¹ Link da BBC de Londres: <<https://www.youtube.com/watch?v=ZxBOMGi7HUU>> acessado em 25/11/2015.

agroindustrial e agregação de valor econômico, mas, sobretudo à omissão em explorar as qualidades bioativas e funcionais das frutas.

Apesar disto, do ponto de vista agroindustrial, observa-se um crescimento na produção e consumo de algumas frutas nativas, a exemplo do açaí. Nos últimos anos, o estado do Pará se destacou por ser o maior produtor nacional de açaí, produzindo 106.562 toneladas ao ano, dado de grande relevância no contexto socioambiental, econômico e social gerando renda para milhares de famílias. A extração da polpa desse fruto resulta em produtos como xaropes, sorvetes, licores (Carneiro et al., 2013) e cosméticos, os quais possuem um expressivo mercado consumidor interno e externo na América do Norte e Europa.

Do ponto de vista alimentício, espécies frutíferas nativas como araçá-boi, ata, cajá e ciriguela obtiveram aceitação satisfatória no paladar dos examinadores, de acordo com um estudo prévio (Souza-Filho et al., 2000), indicando alternativas promissoras no aproveitamento destes frutos na fabricação de néctares (sucos com concentração de 10 a 30 % da polpa), dentre outras possibilidades nutricionais e bioativas.

Considerando que a indústria alimentícia não está apenas interessada no apelo sensorial desses frutos, mas também em seus nutrientes, verifica-se um investimento estratégico na promoção da saúde com o intuito de comercializar produtos que, em quantidades significativas na dieta, desempenham efeitos fisiológicos ou metabólicos e atuam na manutenção das funções do organismo (Vidal et al., 2012).

Do ponto de vista preventivo, estudos epidemiológicos incluem a presença de polifenóis na dieta, inclusive em frutas, como os responsáveis por prevenir o desenvolvimento de doenças crônicas geradas pelo estresse oxidativo como: aterosclerose (processo inflamatório endotelial causado por acúmulo do colesterol “ruim” LDL), doenças cardiovasculares, artrite, diabetes, osteoporose, alguns tipos de câncer, dentre outras enfermidades (Lopez-Varela et al., 2002; Pandey e Rizvi, 2009; Domeneghini e Lemes, 2011). Sendo assim, na intrínseca relação entre alimentos e benefícios para a saúde, as frutas nativas brasileiras podem ser potenciais alvos para consumo e promoção de saúde devido à presença de compostos fenólicos, como flavonoides, antocianinas e estilbenos.

Pesquisas realizadas por Haminiuk et al., (2014) e Infante et al., (2015)² em espécies frutíferas nativas brasileiras como *Eugenia myrcianthes* (ubajaí), *Eugenia uniflora* (pitanga), *Eugenia brasiliensis* (grumixama), *E. pyriformis* (uvaia), identificaram flavonoides como quercetina e miricetina em sua composição. As espécies nativas *Eugenia leitonii* (araça-

²Artigo científico em análise para publicação na PlosOne: Infante, J; Lazarini J.G; Franchin M; Rosalen P.L; Alencar S.M. Antioxidant and anti-inflammatory activities of unexplored Brazilian native fruits.

piranga), *Eugenia involucrata* (cereja do rio grande), *E. myrcianthes* e *E. brasiliensis* apresentaram compostos fenólicos catequinas e epicatequinas em sua composição. Compostos como ácido gálico e antocianinas foram identificados na espécie *E. brasiliensis*. Alguns dos compostos presentes nestas espécies frutíferas foram identificados como sendo os responsáveis por suas atividades antioxidante, antimicrobiana e, particularmente, anti-inflamatória.

Estudos prévios do nosso grupo ainda não publicado (Infante et al., 2015), avaliou-se a atividade anti-inflamatória *in vivo* por meio do teste de migração de neutrófilos induzida por carragenina dos extratos de folha, semente e polpa das espécies *E. brasiliensis*, *E. leitonii*, *E. myrcianthes* e *E. involucrata*. Dentre todos os extratos analisados no estudo, a semente de *E. leitonii* se destacou por apresentar a maior redução do influxo de neutrófilos na cavidade peritoneal de camundongos (62%, quando comparada ao controle carragenina). Esta atividade biológica evidenciada pelos autores na referida espécie, pode estar associada com a presença dos compostos fenólicos citados anteriormente, os quais estão relacionados à capacidade de modular o processo inflamatório no intuito de remover ou destruir o(s) agente(s) agressor(es) de maneira rápida e eficiente.

Nesse contexto, os macrófagos produzem mediadores pró-inflamatórios como as citocinas fator de necrose tumoral – α (TNF- α), interleucina - 1β (IL- 1β), quimiocinas (CXCL1/KC e CXCL2-MIP), o mediador lipídico (leucotrieno B4), óxido nítrico (NO), entre outros, os quais são importantes no processo inflamatório por permitirem defesa do hospedeiro e a remoção do agente flogístico (Ashley et al., 2012). Na modulação da resposta inflamatória, os neutrófilos, principais células da imunidade inata, são quimioatraídos em direção aos mediadores liberados pelos macrófagos e chegam ao sítio inflamatório devido à vasodilatação e ao aumento da permeabilidade vascular. Os neutrófilos interagem com as moléculas de adesão e rolamento (denominadas selectinas dos tipos L-, P- e E-) e moléculas de adesão intercelular (ICAM-1 e VCAM-1) presentes no endotélio, fazendo com que transmigrem para o foco inflamatório sob a influência de quimiocinas, que guiam essas células até o local da injúria (Dinarello et al., 2000; Zhang et al., 2001; Medzhitov, 2008; Ashley et al., 2012; Williams et al., 2011; Sadik & Luster., 2012). Uma vez nos tecidos, os neutrófilos tornam-se ativos com enzimas hidrolíticas (proteínases, catepsinas), espécies reativas de oxigênio (ROS) e nitrogênio, quelantes de vitaminas, que são capazes de combater o processo inflamatório (Medzhitov, 2008). Todavia, a presença dos neutrófilos em quantidades excessivas e constantes, como em uma resposta inflamatória exacerbada, faz com

que os tecidos estejam sempre sob o ataque dessas células, que geram efeitos deletérios e indesejáveis podendo ocasionar a perda da função tecidual.

Estudos apontam que a expressão de certos receptores na superfície dos neutrófilos e a sinalização intracelular desempenham um papel importante na patogênese de doenças como aterosclerose, hipertensão, doença periodontal (Németh e Mócsai, 2012), obesidade, artrite reumatoide, lúpus eritematoso, dentre outras. Assim, a hipótese de que os neutrófilos não atuam apenas como coadjuvantes, mas exercem um papel principal na patogênese de certas doenças é vista como um importante alvo para intervenções terapêuticas (Behling et al., 2004; Wong e Lord, 2004; Mazor et al., 2008; Németh e Mócsai, 2012). Portanto, a busca por novas substâncias bioativas e mecanismos para a terapia anti-inflamatória, que interfiram nos mecanismos acima mencionados torna-se cada vez mais estratégica em abordagens terapêuticas (Mackay, 2008), além obviamente da busca por fármacos com menor efeito adverso que os anti-inflamatórios atuais.

O Brasil ocupa o quarto lugar dentre os países que mais consomem medicamentos, aproximadamente 2 bilhões de caixas por ano, e os anti-inflamatórios, analgésicos e antimicrobianos estão entre as classes farmacológicas de maior consumo (Rios et al., 2013). Apesar do elevado uso dos anti-inflamatórios, e da sua eficácia, é importante ressaltar a necessidade da pesquisa e desenvolvimento de novas moléculas e alvos terapêuticos, que minimizem ou evitem os efeitos adversos resultantes da terapia clássica com anti-inflamatórios como: nefrotoxicidade, vasoconstrição, distúrbios gastrintestinais (náusea, dor gástrica úlceras pépticas) entre outros, e que levam à descontinuação do tratamento (Laveti et al., 2013). Desta forma, embora exista atualmente um número considerável de fármacos anti-inflamatórios para uso médico e odontológico para controle da dor e inflamação, a pesquisa e desenvolvimento de novos fármacos a partir de produtos naturais que apresentem maior eficácia e menores efeitos adversos fazem-se necessários (Kummer e Coelho, 2002; Andrade, 2014).

As frutas nativas brasileiras fazem parte desse cenário na busca por novos padrões moleculares com ação anti-inflamatória que seu mecanismo seja distinto ao da terapia clássica, agregando valores econômicos e sociais na flora nativa brasileira. Dessa maneira, devido à excelente atividade do extrato da semente de *E. leitonii* em reduzir a migração neutrofílica para o foco inflamatório, previamente identificada por Infante et al. (2015), esta fruta nativa foi selecionada para o presente estudo.

Portanto, os objetivos deste estudo foram avaliar o potencial anti-inflamatório, mecanismo(s) de ação e o perfil fitoquímico do extrato da semente de *E. leitonii*, considerado

o mais eficaz na redução do influxo neutrofílico em peritônio de camundongos induzido por carragenina previamente relatada.

2 ARTIGO

Evaluation of the anti-inflammatory activity and phytochemistry of *Eugenia leitonii* D. Legrand, an unexplored Brazilian native fruit³

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ABSTRACT

This study aimed to evaluate the anti-inflammatory activity, mechanism(s) of action and phytochemical profile of the extract from the seeds of *E. leitonii* (EL), an unexplored Brazilian fruit species. EL extract did not affect significantly cell viability, reduced the levels of TNF- α and CXCL2/MIP-2 *in vitro* and *in vivo*, and NF-kB activation in RAW 264.7 macrophages. Mice pre-treated with EL showed significant reduction of influx of neutrophils into their peritoneal cavity; diminished ICAM-1 and P-selectin expression and reduced paw edema. The total phenolic contents yielded 158.74 $\pm$ 3.5 mg GAE/g and anthocyanins, flavonols, phenolic acid and tannins were identified by LC-MS/MS. Thus, EL seeds showed to be a promising source of bioactive compounds with anti-inflammatory potential, acting on pathways of inflammatory mediators and biphasic mechanism in paw edema. Moreover, the extract exhibited content of phenolic compounds higher than that of conventional fruits, which may be related with the anti-inflammatory effects observed.

Keywords: Anti-inflammatory, *Eugenia leitonii*, Native fruits, Phenolic compounds, Cytokines.

Highlights

- This study demonstrates the anti-inflammatory activity and phytochemistry of *Eugenia leitonii*.
- Anti-inflammatory activity was observed *in vitro* and *in vivo*.
- The extract has high concentrations of phenolic compounds.
- The extract exhibited anthocyanins, flavonols, phenolic acid and tannins.
- It may be a promising source of molecules with anti-inflammatory potential.

1 INTRODUCTION

Brazilian native fruit species have been studied for their functional/therapeutic potential as a promising, unexplored source of novel bioactive molecules. With approximately 400 Brazilian species among tree and bushes, the plants of the genus *Eugenia* have been used in folk medicine (Petrovski et al., 2008) due to their relevant biological properties, such as anti-inflammatory (*E. edulis*, *E. brasiliensis*, *E. uniflora*, *E. myrcianthes*, *E. jambolana* and *E. leitonii*), antimicrobial (*E. uniflora*, *E. brasiliensis* and *E. leitonii*), antioxidant (*E. brasiliensis*, *E. involucrata*, *E. myrcianthes*, *E. leitonii* and *E. uniflora*), antidiabetic (*E. malaccense*), anti-arrhythmic (*E. uniflora*), diuretic (*E. brasiliensis*), and vasodilator (*E. uniflora*). These plant species are usually consumed in folk medicine as infusion (Hussein et al., 2003; Fiuza, Saboia-Morais, De Paula, Tresvenzol, & Pimenta, 2008; Petrovski et al., 2008).

The chemical composition of these fruit species commonly include phenolic compounds as quercetin, quercetrin, catechin, epicatechin, anthocyanins, myricetin, gallic acid and others. Regular fruit consumption as a functional food may be related with a reduced risk of developing diseases originating from oxidative stress such as chronic diseases, cardiovascular disorders, cancer, diabetes, and others (Lopez-Varela, Gonzalez-Gross, & Marcos, 2002; Fiuza, Saboia-Morais, De Paula, Tresvenzol, & Pimenta, 2008; Petrovski et al., 2008; Ramirez et al., 2012; Carvalho, Santos, Carvalho, & Souza, 2013; Oliveira, Destandau, Fougère, & Lafosse, 2014).

In a preliminary study performed by our research group, Infante et al. (2015)⁴ evaluated the *in vivo* anti-inflammatory activity of four native fruit species and their respective parts (seeds, leaves and pulps). Among all the samples, the extract from the seeds of *Eugenia leitonii* was found to be the most active one in reducing the influx of neutrophils into the peritoneal cavity of mice. Thus, this extract was selected to be further tested in the present study.

E. leitonii popularly known as “*araça-piranga*” or “*goiabão*” (Raga, 2005), is an unexplored Brazilian native fruit species in extinction and get in part an ecosytem threatened, the Atlantic Forest, and its seeds may be considered an important source of compounds with potential biological properties. In the present study, we investigate the anti-inflammatory

⁴ Data submitted to publication in the journal PlosOne.

activity, mechanism(s) of action and phytochemical profile of the extract from the seeds of *E. leitonii*.

2 MATERIAL & METHODS

2.1 Plant material

E. leitonii (EL) fruits were collected in a local farm (Bello's ranch) (S 23° 23' e W 45° 39') in the city of Paraibuna, São Paulo, Southeastern Brazil, in the months of February and March 2012, under permission of the Brazilian Ministry of Environment (Council for the Administration and Management of Genetic Heritage - CGEN) via the National Council for Scientific and Technological Development (CNPq) [010907/2014-9]. Botanical specimens were deposited in the herbarium of "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba, São Paulo, under voucher number ESA123645. The fruit seeds were separated, lyophilized, weighed and then stored at -18°C.

2.2 Extract preparation

The lyophilized seeds (50 g) were mixtured with a solution of hidroethanolic 80 % (80 : 20, v/v), 400 mL of ethanol (EtOH) and 100 mL of water (H₂O) and then subjected to ultrasound for 30 min at room temperature, in order to obtain the crude extract. The extract was dissolving in saline solution and used in all the experiments also as negative control in the assays. This procedure was repeated three times, then the extract was filtered, evaporated and store at -18 °C until use.

2.3 Experimental animals

Male *Balb/c* albino mice (20 – 25 g), SPF (specific-pathogen free), were purchased from CEMIB/UNICAMP (Multidisciplinary Center for Biological Research, SP, Brazil) and used as experimental animals. The mice were maintained in vivarium under control of temperature (22 ± 2 °C) and humidity (40-60 %), 12 h light/12 h dark cycle, food and water *ad libitum*. The assays were conducted in accordance with the guidelines for the care and use of animals and had prior approval of an Ethics Committee on Animal Research (CEUA UNICAMP, protocol no. 3325-1).

2.4 Anti-inflammatory activity

2.4.1 Cell culture and viability assay

RAW 264.7 macrophage cells were obtained from the cell bank of Rio de Janeiro (ATCC; Rio de Janeiro, Brazil) and cultured in RPMI 1640 medium (Sigma-Aldrich Chemical Co.) supplemented with 10 % fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, streptomycin sulfate and L-glutamina (37 °C, 5 % CO₂). In order to determine the effects of EL extract on cell viability using the methylthiazoletetrazolium - MTT (Sigma-Aldrich Chemical Co.) method, RAW 264.7 cells were seeded in 96-well plates (1 x 10⁶ cells/well) and incubated overnight under the conditions previously described. The cells were pretreated with EL extract at the concentrations of 0.02, 0.2, 2, 20, 200, 300, 400 and 500 µg mL⁻¹ for 24 h. Then MTT solution (0.3 mg mL⁻¹) was added to each well (200 µL) and the plates were incubated for additional 3 h under the same conditions. Then the supernatant was removed and 200 µL of ethanol (etOH) were added to the wells. The absorbance was measured at 470 nm using an ELISA microplate reader (Model 680, Bio-Rad, Hercules, CA, USA) (Xiang et al., 2015).

2.4.2 Cytokine assays *in vitro*

The effects of the EL extract on inflammatory mediators were determined as previously described (Xiang et al., 2015). Briefly, RAW 264.7 cells were cultured, seeded in 96-well plates (2 x 10⁵ cells/well) and incubated overnight (37 °C, 5 % CO₂). The cells were pre-treated with EL extract at the concentrations of 2, 20 and 200 µg mL⁻¹ for 1 h stimulated with lipopolysaccharide (LPS) at 1 µg mL⁻¹, followed by incubation for 6 h under the same conditions. Then the supernatant was collected and the levels of TNF-α, IL-1β and CXCL2/MIP-2 were determined using an ELISA microplate reader according to the protocols supplied by the manufacturers (R&D Systems, Inc). The results are expressed in pictograms mL⁻¹.

2.4.3 NF-kappa B Activation assay

RAW 264.7 macrophage cells were stably transfected with the gene NF-κB-pLUC to express luciferase by the transcription factor NF-κB, as described by Cooper et al. (2010). The cells were seeded in 24-well plates (3 x 10⁵ cell/well) and incubated overnight (37 °C, 5 % CO₂). The cells were pre-treated with EL extract at the concentrations of 2, 20 and 200 µg mL⁻¹ for 30 min and then stimulated with lipopolysaccharide (LPS) at 10 ng mL⁻¹ for 4 h. Then the cells were lysed with lysing buffer and an aliquot of the suspension was mixed with the reaction reagent containing 25 µL of luciferin (Promega, Madison, WI, EUA). The

quantification of luminescence was performed in a microplate reader (Spectra Max M3, Molecular Devices, EUA) with an integration time of 1000 μ s.

2.4.4 Neutrophil migration into the peritoneal cavity of mice

Mice were pretreated with oral administration of EL extract at the single-doses of 30, 100 and 300 mg kg⁻¹. The positive control group received oral dexamethasone at 2 mg kg⁻¹ and the negative group the vehicle saline. After 1 h, an intraperitoneal (i.p.) injection of carrageenan (500 μ g/cavity) was done in all the animals, except for the saline group. The mice were killed 4 h since challenge and their peritoneal cavity was washed with 3 mL of 1 mM PBS/EDTA to obtain the cells suspension. Total and differential counting of leukocytes were carried out according to Denny et al. (2014).

2.4.5 Cytokine assays *in vivo*

Mice were pre-treated with oral administration of EL extract at 300 mg kg⁻¹ in a single dose 1 h before the i.p. administration of the inflammatory stimuli carrageenan (500 μ g/cavity). The negative control group received the vehicle saline. After 3 h, the animals were killed and their peritoneal cavity was washed with 3 mL of 1 mM PBS/EDTA. The levels of TNF- α , IL-1 β , and CXCL2/MIP-2 were determined using an ELISA microplate reader according to the protocols supplied by the manufacturers (R&D Systems, Inc). The results are expressed in picograms (Taktak & Lee, 1991).

2.4.6 Evaluation of ICAM-1 and P-selectin expression by Western blot analysis

Mice were pretreated with oral administration of EL extract at 300 mg kg⁻¹ in a single dose 1 h before i.p. administration of carrageenan (500 μ g/cavity). The negative control group received the vehicle saline. After 4 h, the animals were killed and the proteins of the mesenteric tissue were isolated, quantified and equal amounts of proteins (80 μ g) were transferred to a nitrocellulose membrane (Bio-Rad). A molecular weight standard was run in parallel. The membranes were blocked overnight at 4°C in TBS-T, then incubated with rabbit anti-ICAM-1 (Santa Cruz, CA, USA); (1:500), goat anti-P-selectin (1:1000) and α -tubulin (Santa Cruz CA, USA), which was used as an internal control (1:1000). The membranes were incubated with rabbit and goat anti-IgG conjugated to peroxidase (1:10000) diluted in TBS-T containing 6 % of nonfat milk for 1 h at room temperature. Then, the bands of specific antibody were visualized using a chemiluminescence ECL system (Amersham Biosciences)

and exposed a X ray film for 30 s (Eastman Kodak). Finally, was utilized the software Image J to measure the intensity of the Optical density bands (Franchin et al., 2013).

2.4.7 Carrageenan-induced paw edema

Mice were pretreated with oral administration of EL extract at 300 mg kg⁻¹ in a single dose. The positive control group received orally dexamethasone at 2 mg kg⁻¹ and the negative group received the vehicle saline. After 1 h, the animals received plantar injection of 50 µL of carrageenan (1 mg paw⁻¹) in their left hind paw. Paw volume was measured before (time 0) and after injection of the flogistic agent (1, 2, 3, 4, 5 and 24 h) using a plethysmometer (Ugo Basile, Model 7150, Italy). The edematogenic responses were expressed by subtracting the volume value at time 0 (basal volume) and after the administration of the flogistic agent in the paw in different times Δ (mL) (Denny et al., 2013).

2.5 Chemical analysis

2.5.1 Total phenolic contents

The total phenolic content was determined based on the spectrophotometer method of Folin-Ciocalteu modified by Al-Duais, Muller, Bohm & Jetschke, 2009. Aliquots of 20 µL of EL extract and acid galic standard were added to the wells of microplates containing a solution of 10 % Folin-Ciocalteu. After 5 min, 75 µL of sodium carbonate solution was added to the wells. The results of total phenolic content are expressed in gallic acid equivalent / gram of extract (GAE/g).

2.5.2 LC-MS/MS analysis

First, was weight 10 mg from EL extract and added in 500 µL of methanol and more 500 µL of an aqueous solution of 0.1% formic acid. The LC-MS/MS was performed in the following conditions: The mass spectrometry (MS/MS) system employed for analyte identities conformation analyses was a Quattro triple quadrupole (Micromass, Manchester, UK) equipment fitted with a Z-electrospray (ESI) interface operating on positive and negative ion modes. The temperatures of source block and desolvation gas were set at 100 °C and 350 °C, respectively. Nitrogen was used as both drying (nearly 380 Lh⁻¹) and nebulizing (nearly 38 Lh⁻¹) gas, while argon was used as collision gas. The cone voltages employed during the analyses ranged from 20, 40 or 60 V and the collision energy ranged from 15 to 30 eV among the analytes analyses. For identities confirmation, the analyses were carried out in the

multiple-reaction monitoring (MRM) mode. The ion transitions under MRM mode employed for analytes monitoring. Prepared samples were introduced into the MS/MS system by direct infusion under a flow rate at $10 \mu\text{l min}^{-1}$. The following authentic standards were examined: Cyanidin-3-galactoside, Delphinidin-3-glucoside, Delphinidin-3-pentoside, Malvidin-3-glucoside, Bis-HHDP-glucose, Digalloyl-HHDP-glucose, Ellagic acid, Ellagic acid pentoside, Quercetin hexoside and Quercetrin (Sigma-Aldrich®)

2.6 Statistical analysis

The results were expressed as mean $\pm$ standard deviation (SD) and the differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Results were considered significant when $P \leq 0.05$. To the correlation analysis was applied the Pearson's correlation test and the according the classification: very strong linear correlation $|0.9 \leq r < 1|$; strong linear correlation $|0.6 \leq r < 0.9|$; moderate linear correlation $|0.3 \leq r < 0.6|$ and weak linear correlation $|0.0 \leq r < 0.3|$.

3 RESULTS

3.1 Effects of EL extract on the viability of RAW 264.7 macrophage cells

As seen in Figure 1, EL extract at concentrations ranging from 0.02 to $200 \mu\text{g mL}^{-1}$ did not affect significantly cell viability by the MTT method as compared with the vehicle (cells in culture medium) ($P > 0.05$). Moreover, the concentrations 300, 400 and $500 \mu\text{g mL}^{-1}$ did affect the cell viability. Hence, the concentrations of 2, 20 and $200 \mu\text{g mL}^{-1}$ were used in subsequent assays.

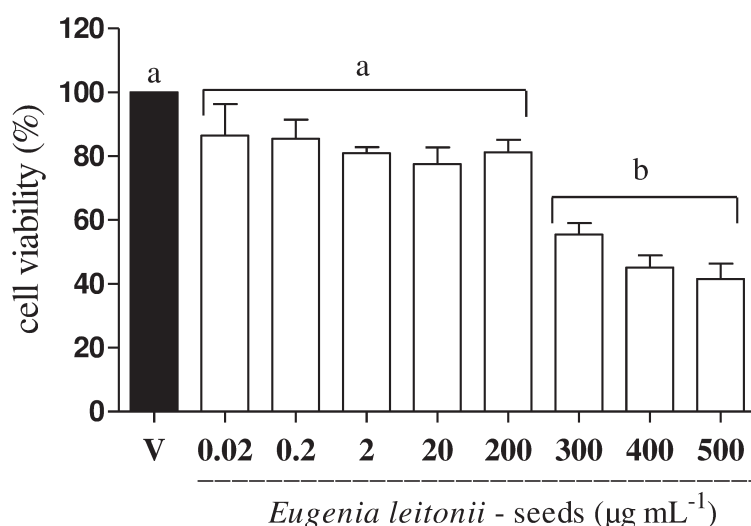


Figure 1: Percent viability of RAW 264.7 cells treated with EL extract at 0.02, 0.2, 2, 20, 200, 300, 400 and 500 $\mu\text{g mL}^{-1}$, and with the vehicle (V) to 24 h. The results were expressed as the mean $\pm$ standard deviation (SD), $n=6$, and the data of the vehicle group were normalized to 100 %. . Different letters indicate statistical significance and equal letters indicate no statistical difference. One-way ANOVA followed by Tukey's post-test, $P < 0.05$. The assay was performed in triplicate

3.2 Cytokine assays *in vitro* and NF-kappa B activation in supernatant

At 200 $\mu\text{g mL}^{-1}$ the extract caused RAW 264.7 cells to significantly decrease the synthesis and/or release of TNF- α and CXCL2/MIP-E (Figure 2A & C) as compared with the LPS-stimulated control group (C+LPS) ($P < 0.05$) and the EL extract did not show statistical difference ($P > 0.05$) as compared with saline grupo (C) (Figure 2A & C). No significant ($P > 0.05$) changes were found in the levels of IL-1 β in any concentration (Figure 2B). Nevertheless, at 2 and 20 $\mu\text{g mL}^{-1}$ the extract did not affect the synthesis and/or release of all inflammatory mediators compared with the negative control (C+LPS) ($P > 0.05$) (Figure 2A, B & C).

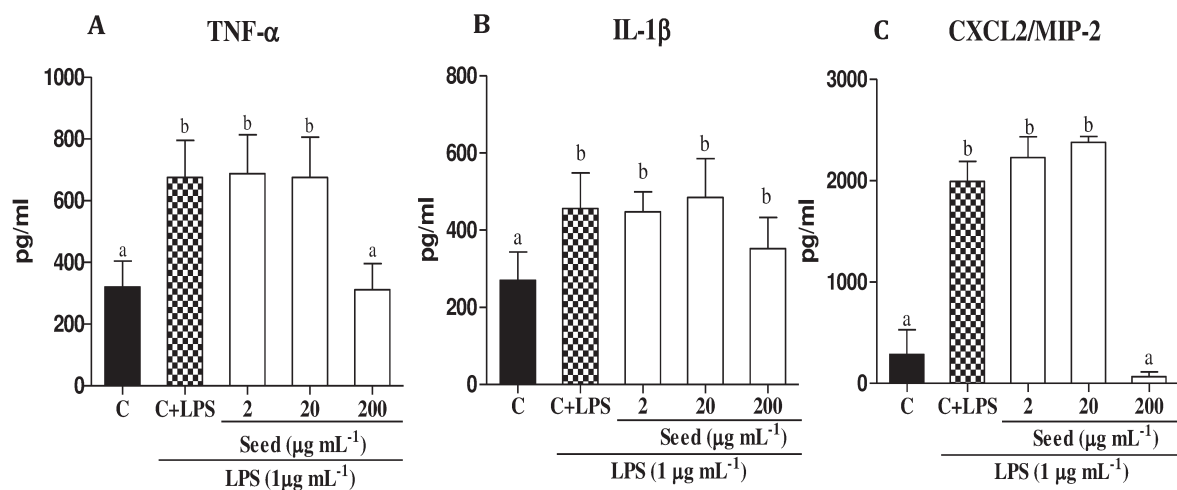


Figure 2: Effect of EL extract (2, 20 and 200 $\mu\text{g mL}^{-1}$) on the synthesis and/or release of TNF- α (A), IL-1 β (B) and CXCL2/MIP-2 (C) in RAW 264.7 macrophage cells. Control groups: (C) cells in culture medium and (C+LPS) LPS-stimulated cells. The EL extract was dissolving with vehicle saline. The results were expressed as mean $\pm$ SD, $n=6$. Different letters indicate statistical significance and equal letters indicate no statistical difference. ANOVA followed by Tukey's post-test, $P < 0.05$.

The ability of EL extract to decrease NF- κ B activation in LPS-treated RAW 264.7 cells was also investigated. As shown in Figure 3, the cells treated with EL extract at 200 $\mu\text{g mL}^{-1}$ had a significant decrease in NF- κ B activation when compared with the negative control group (C+LPS) ($P < 0.05$), however did not reach the NF- κ B level of saline grupo (C) ($P < 0.05$). The extract concentrations of 2 and 20 $\mu\text{g mL}^{-1}$ did not affect NF- κ B activation ($P > 0.05$) compare to C+LPS group.

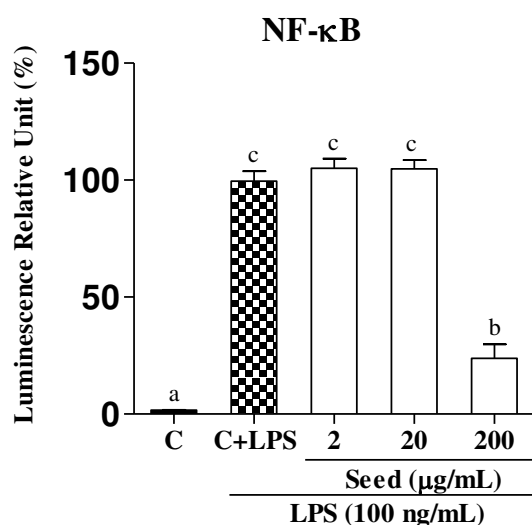


Figure 3: Effects of EL extract (2, 20 and 200 $\mu\text{g mL}^{-1}$) on NF- κB activation in RAW 264.7 macrophage cells. Control groups: (C) cells in culture medium and LPS-stimulated cells (C+LPS). The results were expressed as mean $\pm$ SD, $n=6$. Different letters indicate statistical significance and equal letters indicate no statistical difference. ANOVA followed by Tukey's post-test, $P < 0.05$.

3.3 Cytokine assays *in vivo*

We next studied its effects on the levels of TNF- α , IL-1 β and CXCL2/MIP2 in treated mice (Figure 4). The mice treated with EL extract (300 mg kg^{-1}) had a significant decrease in levels of TNF- α (Figure 4A) and CXCL2/MIP-2 (Figure 4C) when compared with negative control group carrageenan (-) ($P < 0.05$), however the levels of TNF- α and CXCL2/MIP-2 did not reach the saline group (C) ($P < 0.05$). Nevertheless, there was no difference in the levels of IL-1 β (Figure 4B) compared with the carrageenan group ($P > 0.05$).

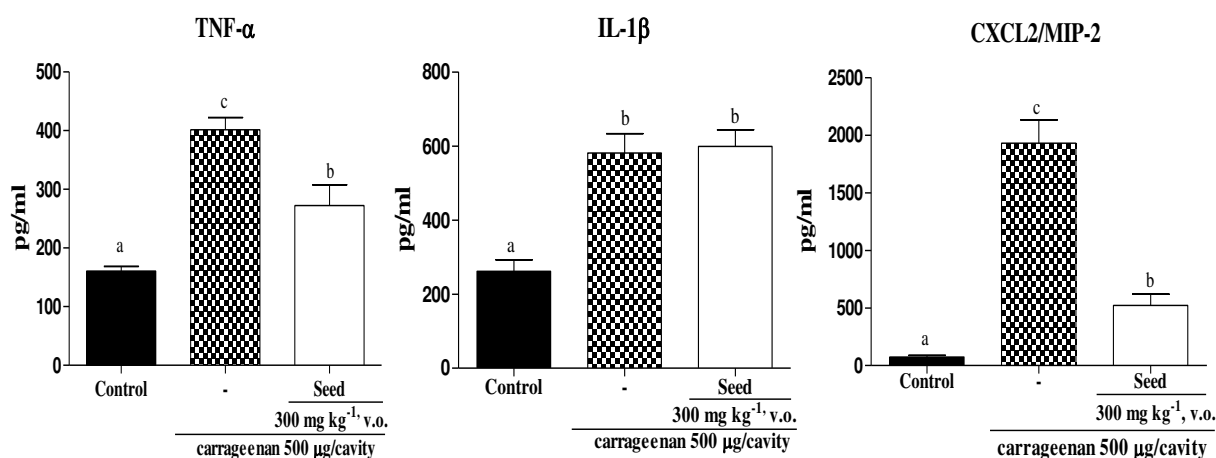


Figure 4: Effects of EL extract on the synthesis and/or release of TNF- α (A), IL-1 β (B) CXCL2/MIP-2 (C) into the peritoneal fluid of mice. The graphs show the control group (Control) treated with the vehicle; carrageenan (-) and EL extract (300 mg kg^{-1}) followed by carrageenan injection. The results were expressed as mean $\pm$ SD, $n=6$. Different letters indicate statistical significance and equal letters indicate no statistical difference. ANOVA followed by Tukey's post-test, $P < 0.05$.

3.4 Neutrophil migration into the peritoneal cavity of mice

We found that the animals pre-treated with the extract showed dose-dependent significantly reduced neutrophil migration into their peritoneal cavity of 31 % (30 mg kg⁻¹), 48 % (100 mg kg⁻¹) and 57 % (300 mg kg⁻¹) as compared with the carrageenan control ($P < 0.05$). At the dose of 300 mg kg⁻¹ the extract was found to be more effective ($P < 0.05$) as compared with other doses (30 and 100 mg kg⁻¹) in reducing neutrophil influx. At the doses (30, 100 and 300 mg kg⁻¹) the extract was not found to be more effective than the positive control dexamethasone (Dexa) and saline group (C) as shown in Figure 5. Pearson's correlation test was applied and according to the classification by Callegari-Jacques (2003), EL extract at 300 mg kg⁻¹ showed strongly linear positive correlation ($r = 0.79$) in a dose-dependent manner ($P < 0.05$).

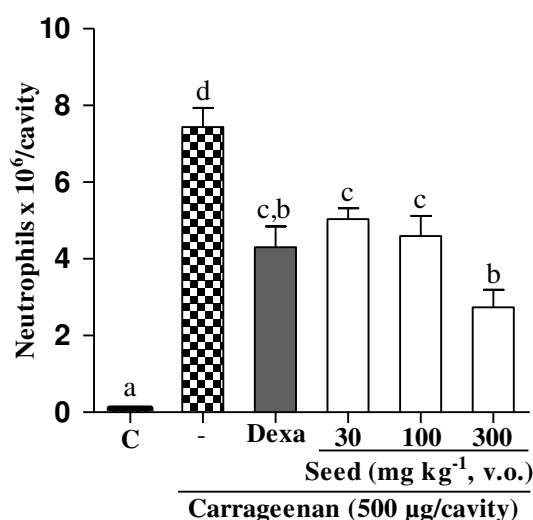


Figure 5: Effect of EL extract on carrageenan-induced neutrophil migration into the peritoneal cavity of mice. Control (C) treated with the vehicle; carrageenan (-); dexamethasone (2 mg kg⁻¹) and EL extract (30, 100 and 300 mg kg⁻¹) followed by carrageenan injection. The results were expressed as mean $\pm$ SD, n=6. Different letters indicate statistical significance and equal letters indicate no significant difference. One-way ANOVA followed by Tukey's post-test, $P < 0.05$.

3.5 Evaluation of ICAM-1 and P-selectin expression by Western blot analysis

The *in vivo* expression of adhesion (ICAM-1) and rolling (P-selectin) molecules was examined to elucidate one of mechanism(s) by which EL extract inhibited neutrophil influx into the peritoneal cavity of mice. The animals were pretreated with the EL extract at the dose of 300 mg kg⁻¹ showed significant decrease in the expression of adhesion and rolling proteins (Figure 6A & B) as compared with the carrageenan group ($P < 0.05$). There was no statistical difference between EL extract and saline used as a control ($P > 0.05$) (Figure 6 A & B).

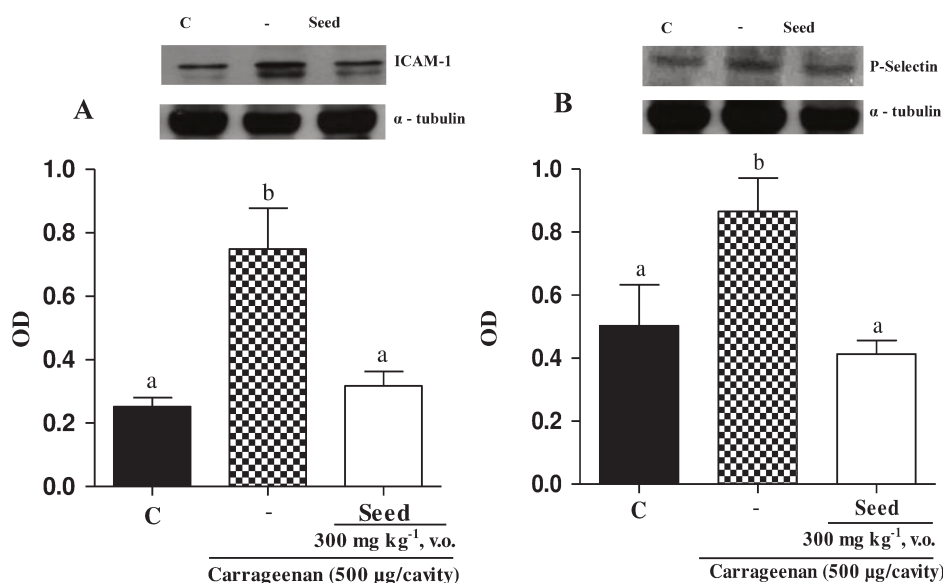


Figure 6: Effect of EL extract on the expression of ICAM-1(A) and P-selectin (B) after injection of carrageenan in the peritoneal cavity of mice. Control (C) treated with the vehicle; carrageenan (-); and EL extract (300 mg kg⁻¹). The results were expressed as mean $\pm$ SD, n=6. Different letters indicate statistical significance and equal letters indicate no significant difference. One-way ANOVA followed by Tukey's post-test, $P < 0.05$.

3.6 Carrageenan-induced paw edema

With the purpose of corroborate the mechanisms of EL extract in reducing the overflow of plasma, a carrageenan-induced paw edema assay was carried out. Table 1 shows the mean volume of edema for experimental and control groups up to 24 h.

Table 1: Effect of oral administration of EL extract on left hind paw after carrageenan injection in mice.

Group	Dose mg kg ⁻¹	Mean edema ΔV mL values $\pm$ SD (% of inhibition)					
		1 h	2 h	3 h	4 h	5 h	24h
Control	-	0.11 $\pm$ 0.02 ^a	0.16 $\pm$ 0.03 ^b	0.17 $\pm$ 0.03 ^b	0.15 $\pm$ 0.02 ^b	0.14 $\pm$ 0.04 ^b	0.09 $\pm$ 0.01 ^b
Dexamethasone	2	0.09 $\pm$ 0.04 ^a (24%)	0.10 $\pm$ 0.05 ^a (42%)	0.10 $\pm$ 0.02 ^a (40%)	0.09 $\pm$ 0.03 ^a (41%)	0.09 $\pm$ 0.03 ^a (35%)	0.07 $\pm$ 0.03 ^a (28%)
EL extract	300	0.06 $\pm$ 0.03 ^a (47%)	0.07 $\pm$ 0.02 ^a (60%)	0.06 $\pm$ 0.02 ^a (64%)	0.08 $\pm$ 0.03 ^a (50%)	0.08 $\pm$ 0.03 ^a (47%)	0.07 $\pm$ 0.03 ^a (28%)

Table 1: The values represent the mean difference of volume of the paw $\pm$ SD, n = 6. $P < 0.05$. In the columns, the different letters indicate statistical significance and equal letters indicate no significant difference. The symbol “%” indicates decrease percentage of edema as compared with the control group (One-way ANOVA followed by Bonfferoni test).

In the present study, the oral administration of EL extract (300 mg kg⁻¹) showed maximal inhibition of edema after 3 h (0.06 $\pm$ 0.02; 64 %) compared with the control group ($P < 0.05$), as shown in Table 1. The positive group, dexamethasone (2 mg kg⁻¹), showed maximal inhibition after 2 h (0.10 $\pm$ 0.05; 42 %) when compared with the control group ($P < 0.05$).

0.05). There was no significant difference between EL extract and dexamethanose with regard to inhibition of paw edema ($P > 0.05$) (Table 1).

3.7 Chemical analysis

3.7.1 LC-MS/MS analysis and total phenolic contents

As the totality of compounds were not identified (data not published), additional LC/MS-MS analysis was done to identify the phenolic compounds in the sample. A total of ten compounds were identified (Table 2) using a mass spectrometer and the total phenolic contents in EL extract were quantified.

Table 2: Chemical compounds identified using LC-MS/MS.

Compounds		Ion molecular [M-H] ⁻ or [M+H] ⁺ (m/z)	MS / MS ion
1	Cyanidin-3-galactoside	449	287
2	Delphinidin-3-glucoside	465	303
3	Delphinidin-3-pentoside	435	303
4	Malvidin-3-glucoside	493	331
5	Bis-HHDP-glucose	783	481, 463, 301
6	Digalloyl-HHDP-glucose	785	633, 615, 301, 275
7	Ellagic acid	301, 303	229, 185, 285, 275, 201
8	Ellagic acid pentoside	433	301
9	Quercetin hexoside	463	301
10	Quercetrin	447	300, 301

In this study were identified by mass spectrometer LC-MS/MS compounds as anthocyanins (1 to 4 compounds), ellagitannins (5 to 8 compounds) and flavonols (9 to 10 compounds). In addition, the total phenolic content was carried out by the Folin-Ciocalteu method and EL extract yielded 158.74 ± 3.5 mg GAE/g.

4 DISCUSSION

Brazil has one of the largest biodiversity in the world, whose fauna and flora have been investigated due to the complexity of chemical substances used for human benefits. Brazilian native fruits have been reported as promising sources of bioactive molecules,

including phenolic compounds as quercetin, epicatechin, catechin, resveratrol and others (Lingaraju et al., 2014). Hence, several studies have associated the consumption of these compounds present in native fruits with the prevention of many chronic disorders generated by oxidative stress (Margetts & Buttriss, 2003; Vita, 2005).

The present study evaluated the anti-inflammatory activity of EL extract using *in vitro* and *in vivo* models. Our findings showed that EL extract did not show cytotoxicity to macrophage cells based on the MTT assay (Figure 1). Figueiroa et al. (2013) investigated the cytotoxicity of the aqueous fraction of the extract from *E. uniflora* and *E. malaccensis* seeds against splenocyte cells. The authors showed that the aqueous fractions of both fruit extracts had no toxicity toward this cell type. These fruit species belong to the same genus of the species investigated herein (*Eugenia*).

Literature shows that EL extract may have modulated these inflammatory mediators by means of intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK), nuclear factor- κ B (NF- κ B), and activator protein-1 AP₁ (c-Jun) (Laveti et al., 2013). Despite that, in the present study, EL extract did not interfere with intracellular signaling pathways to trigger IL-1 β production as the caspases complex entitled inflammasome (Paiva-Oliveira et al., 2012).

Chemokines are chemotactic cytokines present in the inflammatory process that regulate cell signaling with a role in leukocyte trafficking (Tillmann, Bernhagen, & Noels, 2013). The chemokine CXCL2/MIP-2 has been considered an important factor to neutrophils chemotaxis into the inflammatory site which is regulated by associated kinases protein (GRK2).

In order to check a likely intracellular signaling pathway through which EL exerts its anti-inflammatory effects, we evaluated NF- κ B activation in macrophages (Figure 3). NF- κ B is a determining factor to activation of genes encoding proteins that modulate the inflammatory process, for instance, cytokines (TNF- α , IL-6), chemokines (CXCL2/MIP-2), and rolling and adhesion molecules on the surface of endothelial cells (Paur et al., 2010; Donepudi et al., 2012). Hence, the modulation of NF- κ B may be a potential target to anti-inflammatory therapy and EL extract was found to affect this process.

In vivo assays in mice were performed to confirm the anti-inflammatory potential observed *in vitro* for EL extract. Mice that received the extract had decreased levels of the cytokine TNF- α and the chemokine CXCL2/MIP-2 (Figure 4). Therefore, the findings on the reduction in the levels of inflammatory mediators observed *in vitro* (Figure 2) corroborate those of the *in vivo* assay (Figure 4).

The inflammatory mediators TNF- α and CXCL2/MIP-2 are important components in the modulation of the inflammatory response. Once released by resident macrophages, these mediators activate primarily the nuclear factors of transcription and subsequently increase the expression of adhesion (ICAM-1) and rolling (P-selectin) molecules to promote neutrophil influx toward inflamed tissues (Xiang et al., 2015). The assay of neutrophil migration into the peritoneal cavity of mice assesses neutrophil influx into the inflammatory focus in an acute inflammation under carrageenan stimuli. Other parameters may also be investigated, as the synthesis and/or release of inflammatory mediators (Xiang et al., 2015). In our study, the highest extract dose had better activity in decrease of neutrophil migration into the peritoneal cavity of animals (Figure 5) as compared with other doses. Such decrease in the influx of defense cells can be linked with the reduced levels of inflammatory mediators and NF- κ B activation.

Thus, to elucidate by which mechanisms EL extract interferes with the influx of inflammatory cells, we assessed the expression of adhesion (ICAM-1) and rolling (P-selectin) molecules in mice (Figure 6). These molecules are fundamental to neutrophil and monocyte internalization to the inflamed tissue. In addition, the inflammatory mediators TNF- α , IL-1 β , and CXCL2/MIP-2 are related with increased influx of neutrophils in from the bloodstream to the inflammatory focus, besides promoting chemotaxis of defense cells resulting in tissue damage (Denny et al., 2013).

We point out that the cells pre-treated with EL extract, as shown in Figure 2, matched the levels of control (cells in culture medium). In the neutrophil migration test (Figure 5), there was no difference compared with the positive control, dexamethasone, which is considered a gold standard drug. Therefore, despite being a complex mixture of phytochemical compounds EL extract reached the same parameters of the positive group. In addition, the group pretreated with EL extract showed reduced expression of rolling and adhesion molecules similarly to the basal levels of the saline group (Figure 6).

Finally, we verify by which mechanisms EL extract interferes with vascular permeability, a carrageenan-induced paw edema assay was carried out (Table 1). Carrageenan is a polysaccharide widely used as a flogistic agent to promote the release of histamine and serotonin from mast cells in the initial phase (1-2 h) after injection of carrageenan and prostaglandins; and lysosomes and proteases in the second phase (3-4 h) after injection of carrageenan (Winter, Risley, & Nuss, 1962; Crunkhon & Meacok, 1971). The results of our study showed that mice pretreated with EL extract had paw edema significantly decreased

from 2 h until 24 h, suggesting that EL extract inhibited the edematogenic effect by means of decrease of histamine, serotonin and prostaglandins.

The dexamethasone used as positive control is a similar endogenous cortisol, interacting with intracellular receptors and promoting inhibitory effect from transcription factors of several genes involved in inflammatory response, resulting in decrease synthesis of cytokines, chemokines, prostaglandins, nitric oxide and others mediators. It is interesting to verify that EL extract may be acting in similar mechanism way to the glucocorticoid once that treat moisture of chemical substances as extract crude and may be a promising source to new agente to prevent and/or therapereutic (Anti, Giorgi, & Chahade, 2008).

Likewise, Schapoval, Silveira, Miranda, Alice, & Henriques (1994) showed that rats pretreated orally with the extracts from the leaves of *Eugenia uniflora* at 300 mg kg⁻¹ showed a reduction in carrageenan-induced paw edema from 1 h until 4 h. Also, El-Shenawy (2009) showed the rats pretreated orally with the extract from the pericarp of *Eugenia jambolana* at 500 mg kg⁻¹ showed significantly diminished paw edema from 1 h until 4 h.

The anti-edematogenic effect and anti-inflammatory activity founded for EL extract may have been modulated by flavonols, primariially quercetin and quercetrin; anthocyanin, such as cyanidin, malvidin, delphinidin; and polyphenols as ellagic acid, gallic acid present in the extract composition. Several phenolic compounds were identified in the phytochemical analysis, among which the polyphenols quercetin and ellagic acid could be a responsible for the biological activity observed (Séfora-Souza & Angelis-Pereira, 2013).

Anthocyanins such as cyanidin-3-galactoside, delphinidin-3-glucoside, and malvidin-3-glucoside, are flavonoids responsible for the food pigments. A number of studies have reported their biological activities, including anti-inflammatory, cardiovascular, vasoprotective, and others (Wei et al., 2011). Also, Massarioli, Oldoni, Moreno, Rocha, & Alencar, (2013) found phenolic compounds in crude extract of *Eugenia uniflora* L. such as caffeic acid, gallic acid and quercetin-3-Dgalactoside. In the same species, Oliveira et al. (2014) showed the presence the ellagic acid, kaempferol, quercetrin and quercetin with expressive antioxidant capacity.

The ellagitannins, founded in the EL extract, after the consumption, are hydrolyzed arising in ellagic acid (Piwowarski, Kiss, Granica, & Moeslinger, 2015). The ellagic acid has antioxidant and anti-inflammatory properties, and the literature has shown it to be a promising polyphenol to treat airways disease (Alves et al., 2013). Ellagitannins possess well known antioxidant and anti-inflammatory bioactivity (Sanches-González, Ciudad, Noé & Izquierdo-Pulido, 2015). In a study, El-Shitany, El-Bastawissy, & El-desoky (2014) showed that rats

pretreated intraperitoneally with the ellagic acid (100 mg kg^{-1}) showed a reduction in carrageenan-induced paw edema from 1 h until 4 h. Also, the authors showed after the treatment intraperitoneally the level of TNF- α and the NF- κ B mRNA expression were decrease in the soft tissues of the rat hind paws.

Quercetin, a flavonoid of the flavonol subclass, has well known biological properties as antioxidant, anti-inflammatory and anti-atherogenic (reducing the risk of cardiovascular diseases by preventing the oxidation of lipoproteins) (Séfora-Souza & Angelis-Pereira, 2013); anti-inflammatory and antinociceptive, decreasing the levels of the inflammatory mediators NO, PGE₂, TNF- α , IL-1 β and others *in vivo* e *in vitro* (Comalada et al., 2005; Denny et al., 2013). Flavonols have also been proven to promote down-regulation in NF- κ B activity (Comalada et al., 2005).

Denny et al. (2013) found phenolic compounds such as epicatechin, myricetin and mainly quercetin in the pomace extract of *Psidium guajava* (guava), which belongs to the same family as *Eugenia leitonii* (Myrtaceae). The authors associated the presence of these compounds with a decrease of inflammatory and nociception mediators (TNF- α and IL-1 β) contributing to anti-inflammatory and antinociceptive properties.

Given the above, EL extract includes several of the aforementioned compounds in its composition, which could potentially account for the functional properties of this Brazilian native fruit and these activities from EL extract may suggest a synergistic effect between the phenolic compounds in the mixture.

The total phenolic content was quantified by the Folin-Ciocalteu method and comparatively, fruits of the same genus have a range of 120 mg AG/g up to 230 mg GAE/g in species as *Eugenia uniflora* (purple variety) and *Eugenia jambolana* (De Lima, Melo, & Lima, 2002; Figueiroa et al., 2013). Interestingly, pulps of well known fruits which are highly consumed, for instance, mango, pineapple, passion fruit, and melon, have phenolic contents between 1.26 and 5.60 mg GAE/g (Martinez et al., 2012), concentration lower than that found in our study for EL extract.

Vieira, Souza, Mancini-Filho, & De Lima (2011) quantified the total phenolic content of the pulps extract in some small Brazilian fruits like acerola $449.63 \pm 10.24 \text{ mg GAE/g}$, bacuri $7.23 \pm 0.08 \text{ mg GAE/g}$, cajá $6.62 \pm 0.90 \text{ mg GAE/g}$, cashew nut $165.07 \pm 4.10 \text{ mg GAE/g}$, guava $20.21 \pm 1.95 \text{ mg GAE/g}$ and tamarindo $23.35 \pm 0.21 \text{ mg GAE/g}$ and the seeds of EL showed lower total phenolic content only as compared with acerola and Guava. These dates not only encourage the research with Brazilian native fruits but also suggest that

minimal consumption of EL may have greater benefits than those of well-known common fruits.

It is important to highlight that all the results in this study concern a native fruit species (*Eugenia leitonii*), particularly its seeds, which brings novelty and originality to international scientific literature.

Taken altogether, this study showed that a native fruit from Brazil has bioactive compounds with anti-inflammatory activity *in vitro* and *in vivo*. Native fruits may be a new source of compounds with anti-inflammatory potential which can foster the agribusiness, pharmaceutical and food industry resulting in the development of new products or pharmaceutical inputs for human health.

5 CONCLUSION

The EL extract, a genuine Brazilian species founded in Atlantic Forest, an ecosystem threatened, showed to be a promising source of bioactive compounds with anti-inflammatory potential, acting on pathways of inflammatory mediators including rolling and adhesion molecules, nuclear factor and exhibit anti-edematogenic effect in the paw edema. The EL extract exhibited content of phenolic compounds (flavonoids and anthocyanins) higher than that of traditional fruits, which may be related with the observed anti-inflammatory effects, allowing its use to enrich foods.

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3 CONCLUSÃO

Assim, essa espécie genuinamente brasileira encontrada na mata atlântica, um ecossistema ameaçado, demonstrou ser uma promissora fonte de compostos bioativos com potencial anti-inflamatório, atuando sobre vias de mediadores inflamatórios envolvendo moléculas de adesão e rolamento, e exibiu efeito anti-edematogênico no edema de pata. O extrato de EL exibiu um teor de compostos fenólicos (flavonoides e antocianinas) maior do que aos frutos tradicionais, que podem estar relacionados com o efeito anti-inflamatório observado possibilitando seu uso para enriquecer alimentos.

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⁵ De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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PROC. Nº 01P-3736/2002

INTERESSADA : COMISSÃO CENTRAL DE PÓS-GRADUAÇÃO (CCPG)

ASSUNTO : NORMAS SOBRE O FORMATO E A IMPRESSÃO DE DISSERTAÇÃO
E/OU TESES (INFORMAÇÃO CCPG/001/2015)

DELIBERAÇÃO CCPG-Nº 284/2015

A COMISSÃO CENTRAL DE PÓS-GRADUAÇÃO DA UNIVERSIDADE ESTADUAL DE CAMPINAS, em sessão realizada em 09/09/2015, tomou ciência e aprovou, por unanimidade, à alteração da redação da Informação CCPG/001/2015, que trata da regulamentação das normas sobre o formato das dissertações de mestrado e teses de doutorado.

Encaminhe-se às CPG's, Diretoria Acadêmica (DAC), Biblioteca Central (BC) e à Gráfica.

CCPG, 09 de setembro de 2015.


Profª. Drª. RACHEL MENEGUELLO
Presidente
Comissão Central de Pós-Graduação

¹² Universidade Estadual de Campinas. Pró-Reitoria de Pós-graduação. Comissão Central de Pós-Graduação. Informação CCPG 001/2015. Campinas: Unicamp; 2015 [acesso 2015 Nov 30]. Disponível em: http://www.prog.unicamp.br/arqpdfnormas/infccpg001_2015.pdf.

ANEXO 2 - Certificado do Comitê de Ética em Animais

CEUA/Unicamp

**Comissão de Ética no Uso de Animais
CEUA/Unicamp****CERTIFICADO**

Certificamos que o projeto "**Prospecção de atividade anti-inflamatória em espécies frutíferas nativas do Brasil em camundongos da linhagem Balb/c**" (protocolo nº **3325-1**), sob a responsabilidade de **Prof. Dr. Pedro Luiz Rosalen / Josy Goldoni Lazarini**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA, SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **17 de março de 2014**.

Campinas, 17 de março de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

ANEXO 3 – Comprovante autorização do Conselho de Gestão do Patrimônio Genético (CGEN)

 Autorização de Acesso e de Remessa de Componente do Patrimônio Genético O Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, nos termos Deliberação 246/2009, do Conselho de Gestão do Patrimônio Genético, autoriza a instituição identificada no verso deste documento a acessar e remeter componente do Patrimônio Genético com a finalidade de pesquisa científica. Brasília, 18 de Dezembro de 2014 Marcelo Marcos Moraes Diretor de Ciências Agrárias, Biológicas e da Saúde PO 161/2010	Processo: 010907/2014-9 Validade: 03/02/2015 a 03/02/2017 Instituição: UNIVERSIDADE DE SAO PAULO CNPJ: 630.255.300/0001-04 Pesquisador: Severino Matias de Alencar CPF: 550.118.024-34 RG: 3682343 - SSP / PE <i>Para visualizar a versão digital da Autorização de Acesso e de Remessa de Componente do Patrimônio Genético, V.Sa. poderá utilizar a ferramenta disponibilizada pelo CNPq para esse fim na página</i> http://servicosweb.cnpq.br/visualizador/ e informar o número do protocolo 3652670898903851 para recuperá-la do banco de dados do CNPq
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ANEXO 4 – Comprovantes de participações em congressos de Sociedades Científicas

4.1 – Sociedade Brasileira de Pesquisa Odontológica (SBPqO)

05/07/2015

SBPqO



Nº Inscrição: Nº Trabalho: 1678

Nome: Josy Goldoni Lazarini **Categoria:** Sócio Aspirante

Área Relacionada: 3 - Fisiologia / Bioquímica / Farmacologia **Modalidade:** Painel Aspirante

Descritores: Anti-Inflamatórios, citotoxicidade, Frutas

Avaliação da atividade anti-inflamatória e citotóxica de *Eugenia brasiliensis* (grumixama)

Lazarini JG*, Franchin M, Denny C, Infante J, Alencar SM, Rosalen PL

Ciências Fisiológicas - Faculdade De Odontologia - UNICAMP. Tel.: 25325367. E-mail: josy662@hotmail.com

O objetivo desse estudo foi avaliar a atividade anti-inflamatória, mecanismo de ação e citotoxicidade da *Eugenia brasiliensis* (grumixama), uma espécie frutífera nativa do Brasil. Extratos hidroetanólicos (80:20, v/v) da *E. brasiliensis* (polpa, folha e semente) foram avaliados por: migração neutrofílica induzida por carragenina (camundongos, n=6, CEUA#3325-1), citotoxicidade celular (MTT) e determinação de TNF- α e CXCL2-MIP em células RAW 264.7. O extrato de menor citotoxicidade foi avaliado quanto ao edema de pata induzido por carragenina. A migração neutrofílica reduziu significativamente com os extratos de polpa ($5,6 \pm 2,1$), folha ($5,8 \pm 2,5$) e semente ($6,6 \pm 1,4$) da *E. brasiliensis*, comparados ao controle negativo ($11,1 \pm 1,4$) ($p \leq 0,05$). A citotoxicidade do extrato da polpa foi insignificante ($p > 0,05$), entretanto os demais extratos reduziram a viabilidade celular ($100 \mu\text{g/ml}$, $p \leq 0,05$). O extrato da semente diminuiu ($p \leq 0,05$) TNF- α e CXCL2-MIP, enquanto os demais extratos reduziram TNF- α comparados ao controle ($p \leq 0,05$). Finalmente, o ensaio do edema de pata demonstrou que a polpa, nas doses de 30mg/kg, 100mg/kg e 300mg/kg, apresentou inibição máxima do edema na 2ª, 4ª e 1ª hora ($0,06 \pm 0,02$; $0,11 \pm 0,02$ e $0,07 \pm 0,03$, respectivamente para doses e hora; $p \leq 0,05$), quando comparados ao controle negativo ($p \leq 0,05$).

Concluindo, a *E. brasiliensis* apresentou atividade anti-inflamatória provavelmente por mecanismo bifásico sendo a polpa menos citotóxica. Assim, sugere-se que *E. brasiliensis* apresenta compostos bioativos agregando valores a espécies frutíferas brasileiras.

(Apoio: FAPESP Nº 2013/26251-0)

4.1 – Federação de Sociedades de Biologia Experimental - FeSBE

Gerado em: 19-05-2015 09:08:56 - XXX Reunião Anual da FeSBE

PROSPECÇÃO DE ATIVIDADE ANTI-INFLAMATÓRIA E CITOTOXICIDADE DE ESPÉCIES FRUTÍFERAS NATIVAS DO BRASIL.

Lazarini, J. G. , Franchin, M. , Denny, C. , Infante, J. , Alencar, S. M. , Rosalen, P. L. ,

Ciências fisiológicas - FOP

Agroindústria, Alimentos e Nutrição - ESALQ

Introdução:

Na busca por substâncias bioativas alternativas, as espécies frutíferas nativas brasileiras são uma nova fonte a ser exploradas, principalmente pelo conteúdo de compostos fenólicos que apresentam. Dentre estes compostos destacam-se os flavonoides com ações antioxidante, anti-inflamatória e outras. Assim, diante da diversidade da flora brasileira e escassez de estudos a cerca de frutas nativas, é relevante investir em pesquisas com o intuito de caracterizar e identificar novas substâncias com alvos terapêuticos, além de agregar valor às espécies vegetais estudadas.

ANEXO 5 – Comprovante de submissão do artigo à revista científica internacional

Submission Confirmation



Journal of Functional Foods (fshahidi@mun.ca) Adicionar aos contatos 05/01/2016 ▶

Para: josy662@hotmail.com ✉

Full Length Article

Dear Miss. Josy Goldoni Lazarini,

We have received your article "Evaluation of the anti-inflammatory activity and phytochemistry of Eugenia leitonii, an unexplored Brazilian native fruit" for consideration for publication in Journal of Functional Foods.

Your manuscript will be given a reference number once an editor has been assigned.

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